Title Page

Study Report for Task Order No. UIC-14

IN VITRO MUTAGENICITY TESTING OF WR6026 HYDROCHLORIDE

Sponsor: US Army Medical Materiel Development Activity

Contract Number: DAMD17-92-C-2001

Test Article: WR6026 Hydrochloride

Principal Investigator

Barry S. Levine, D.Sc., D.A.B.T.

Study Directors

Richard H. C. San, Ph.D. (Microbiological Associates, Inc.)
Patrick T. Curry, Ph.D. (Microbiological Associates, Inc.)

Completion of Testing Date

Performing Laboratory

Microbiological Assoicates, Inc. - Subcontract
Toxicology Research Laboratory (TRL)
University of Illinois at Chicago (UIC)
Department of Pharmacology
1940 W. Taylor St.
Chicago, IL 60612-7353

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WR6026 Hydrochloride was tested for point mutations and chromosomal aberrations in three *in vitro* mutagenicity tests. WR6026 Hydrochloride was shown to be negative in the Ames Test, the Mouse Lymphoma Assay, and the Chromosome Aberration Test using Chinese Hamster Ovary cells. Accordingly, the drug does not appear to represent a genotoxic hazard, and follow-up studies are not warranted.

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IN VITRO MUTAGENICITY
TESTING OF WR6026 HYDROCHLORIDE

Sponsor: US Army Medical Materiel
Development Activity
Fort Detrick
Frederick, MD 21701-5014

Representative: George J. Schieferstein, Ph.D.

Test Article: WR6026 Hydrochloride

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.
9900 Blackwell Road
Rockville, MD 20850

as a subcontract to:

TOXICOLOGY RESEARCH LABORATORY (TRL)
University of Illinois at Chicago (UIC)
Department of Pharmacology
1940 W. Taylor St.
Chicago, IL 60612-7353

_____ _____

Barry S. Levine, D.Sc., D.A.B.T. Date
Principal Investigator

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3 Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells Report 3-1

Contract No.: DAMD17-92-C-2001
Task Order No.: UIC-14

1. SUMMARY

WR6026 Hydrochloride was tested for point mutations and chromosomal aberrations in three *in vitro* mutagenicity tests. WR6026 Hydrochloride was negative in the Ames Test, the Mouse Lymphoma Assay, and the Chromosome Abberation Test using Chinese Hamster Ovary cells.

2. INTRODUCTION

The purpose of this project was to study the *in vitro* mutagenic potential of WR6026 Hydrochloride using the following assay systems.

1. Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

   This test evaluates the mutagenic potential of a test article (and/or its metabolites) by measuring its ability to induce back mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of rat hepatic microsomal enzymes.

2. L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

   This assay tests for specific locus mutations at the thymidine kinase (TK) locus of cultured L5178Y TK+/- mouse lymphoma cells in the presence and absence of rat hepatic microsomal enzymes.

3. Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

   This assay evaluates clastagenic potential based upon the ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

The studies reported herein were conducted at Microbiological Associates, Inc., and the individual study reports are contained in Appendices 1 - 3.

3. RESULTS AND DISCUSSION

The results of the three assays to assess the *in vitro* mutagenicity of WR6026 Hydrochloride are summarized in Table 1. WR6026 Hydrochloride was shown to be negative in the Ames Salmonella Test, the Mouse Lymphoma Assay and the Chromosome Abberations Test in CHO cells.

4. CONCLUSION AND RECOMMENDATIONS

On the basis of the present studies, WR6026 Hydrochloride was negative in all three *in vitro* mutagenicity tests. Accordingly, the drug does not appear to represent a genotoxic hazard, and follow-up studies are not warranted.

Contract No.: DAMD17-92-C-2001
Task Order No.: UIC-14

Table 1

IN VITRO MUTAGENICITY TESTING OF WR6026 HYDROCHLORIDE

Summary of Results

Assay	Doses Tested (ug base/ml)*	Results
Ames test		
-S9	10 - 3333	-
+S9	10 - 3333	-
Mouse lymphoma assay		
-S9	1.0 - 20	-
+S9	0.2 - 0.75	-
Chromosal aberrations in CHO cells test		
-S9	4 - 30	-
+S9	0.8 - 6	-

S9 = Metabolic Activation System (rodent liver 9000 g fraction)
- = Negative

APPENDIX 1

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST) REPORT

DRAFT

DRAFT REPORT II

Study Title

SALMONELLA PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Test Article

WR6026 Dihydrochloride

Sponsor Project Number

UIC-14

Authors

Richard H.C. San, Ph.D.
David L. Pugh, B.S.

Study Completion Date

(Pending Final Report)

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

Laboratory Study Number

G94BT87.501

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

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STATEMENT OF COMPLIANCE

The *Salmonella* Plate Incorporation Mutagenicity Assay (Ames Test) of test article **WR6026 Dihydrochloride** the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 792 and 40 CFR 160, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

RS — 3/2/95

Richard H.C. San, Ph.D.
Study Director

Date

QUALITY ASSURANCE STATEMENT

DRAFT

Study Title: SALMONELLA PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Study Number: G94BT87.501

Study Director: Richard H. C. San, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 14 DEC 94, TO STUDY DIR 14 DEC 94, TO MGMT 14 DEC 94
PHASE: PROTOCOL REVIEW

INSPECT ON 22 DEC 94, TO STUDY DIR 27 DEC 94, TO MGMT 28 DEC 94
PHASE: STRAIN CHARACTERIZATION

INSPECT ON 25 JAN 95, TO STUDY DIR 25 JAN 95, TO MGMT
PHASE: Draft Report

INSPECT ON 02 MAR 95, TO STUDY DIR 02 MAR 95, TO MGMT 02 MAR 95
PHASE: Draft to Draft II Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

CC — 3-2-95

Claire L. Courtemanche
QUALITY ASSURANCE — DATE

DRAFT

SALMONELLA PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

DRAFT REPORT II

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR6026 Dihydrochloride**

Test Article Bottle No.: **BK01845**

Test Article Purity: **>99.4% (Provided by Sponsor)**

Test Article Mole Fraction: **0.825 (Provided by Sponsor)**

Sponsor Project No.: **UIC-14**

MA Study No.: **G94BT87.501**

Test Article Description: **light yellow powder**

Storage Conditions: **-20±5°C; protected from exposure to light**

Test Article Receipt: **12/09/94**

Study Initiation: **12/13/94**

Study Director: RS 3/2/95
Richard H.C. San, Ph.D. Date

Associate Study Director: _____
Valentine O. Wagner, III, M.S. Date

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SUMMARY

The test article, WR6026 Dihydrochloride was tested in the *Salmonella* Mutagenicity Assay using tester strains TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases using the plate incorporation method. The first phase, the dose range-finding study, was used to establish the dose range for the mutagenicity assay. The second phase, the mutagenicity assay, was used to evaluate the mutagenicity of the test article.

In the dose range-finding study, the maximum dose tested was 6050 μg per plate. This dose was delivered to the test system as a clear solution in water. The results of the dose range-finding study indicate that no precipitate but toxicity was observed. Therefore, the maximum dose that was plated in the mutagenicity assay was 3333 μg per plate.

In the mutagenicity assay, no positive responses were observed with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9. In addition, no precipitate but toxicity was observed. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation[a] and Dose Range Tested (μg/plate)									
	TA98		TA100		TA1535		TA1537		TA1538	
	Low	High	Low	High	Low	High	Low	High	Low	High
None	-		-		-		-		-	
	10	3333	10	3333	10	3333	10	3333	10	3333
Rat	-		-		-		-		-	
	10	3333	10	3333	10	3333	10	3333	10	3333

[a] - = negative, + = positive (maximum fold increase)

In conclusion, the results indicate that under the conditions of this study, test article **WR6026 Dihydrochloride** did not cause a positive response in the *Salmonella* Plate Incorporation Mutagenicity Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of S9 activation. This test system has been shown to detect a diverse group of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The ability to induce mutation is indicative of a chemical's genotoxic potential.

MATERIALS AND METHODS

Media and Reagent Preparation

Note: All references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System at MA unless otherwise indicated.

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 μM each. If the top agar was not used with S9 or Sham mix, 25 ml of water were added for each 100 ml of minimal top agar.

Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq$-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100. The microsomal enzyme mixture (S9 mix) was prepared immediately before its use and contained, 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use.

Test System

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a wide range of chemical classes.

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames *et al.* (1975). Tester strains in use at Microbiological Associates, Inc. (MA) were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Genotype of the Strains Used for Mutagen Testing
Figure 1

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537	TA1538	*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

The tester strains contain, in addition to a mutation in the histidine operon, two additional mutations that enhance their sensitivities to some mutagenic compounds. The *rfa* wall mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals, such as those containing large ring systems that would otherwise be excluded by a normal cell wall. The second mutation is a deletion in the *uvr*B gene that results in a deficient DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, tester strains containing this deletion also require the vitamin biotin for growth.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor) that further increases the sensitivities of these two strains to some mutagens. It has been suggested that this plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations.

Frozen Permanent Stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing away approximately 1.5 ml aliquots in glass vials. Frozen Permanent Stocks were stored at ≤-70°C. Master plates were prepared by streaking each tester strain from a frozen permanent onto

minimal medium supplemented with histidine (260 μM), biotin (3 μM) and, for strains containing the R-factor, ampicillin (25 μg/ml). Master plates were incubated at 37$\pm$2°C for 24 to 48 hours and stored at 4$\pm$2°C.

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 ml of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37$\pm$2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of approximately 10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

On the day of their use in the mutagenicity assay, all tester strain cultures were checked for the correct genotype. The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. The deletion in the *uvr*B gene was confirmed by demonstration of sensitivity to ultraviolet light. The presence of the pKM101 plasmid was confirmed by demonstration of resistance to ampicillin. Spontaneous reversion frequencies in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

The test system was exposed to the test article via the plate incorporation methodology described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This methodology has been shown to detect many classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976). The assay was performed in two phases. The first phase, the dose-range finding study, was used to establish the dose-range over which the test article would be assayed. Ten dose levels of the test article were plated, one plate per dose, with an overnight culture of TA100 on selective minimal agar in both the presence and absence of rat S9 activation[a]. The second phase, the mutagenicity assay, was used to evaluate the mutagenicity. A minimum of five dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA1537 and TA1538 in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in triplicate.

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[a]In deviation from the protocol, the top dose tested in Experiment A1 was 6050 μg/plate rather than 5000 μg/plate. This deviation was the result of a calculation error. Since this deviation did not affect the integrity of the data or the validity of the conclusion, the Study Director has accepted the data generated in this experiment.

Test and Control Articles

The test article, WR6026 Dihydrochloride, was received by Microbiological Associates, Inc. on 12/09/94 and was assigned the code number 94BT87. The test article was characterized by the Sponsor as a light yellow powder that should be stored at -15°C to -20°C. No expiration date was provided. Upon receipt, the test article was described as a light yellow powder and was stored at -20±5°C, protected from exposure to light. The vehicle used to deliver WR6026 Dihydrochloride to the test system was water, (CAS# 7732-18-5), distilled, purchased from Gibco. The dosing solutions were adjusted to compensate for the mole fraction (0.825 free base) of the test article. Aliquots of dosing solution preparations were returned to the Sponsor for chemical analysis. Since the test article binds to glass, all test article dilutions and dosing procedures were performed with plastic labware.

Positive controls plated concurrently with the assay are listed in Figure 2.

Positive Controls
Figure 2

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1537	-	2-aminoanthracene	1.0
	-	9-aminoacridine	75
TA1538	-	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0

Source and Grade

9-aminoacridine (CAS #134-50-9), Sigma Chemical Co., 98% pure
2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98 % pure
sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade

To determine the sterility of the test article, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay. To determine the sterility of the S9 or Sham mix, a 0.5 ml aliquot of each was plated on selective agar.

Plating and Scoring Procedures

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in Microbiological Associates, Inc.'s Microbial Mutagenesis Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half (0.5) milliliter of S9 or Sham mix, 100 μl of tester strain and 50 μl of vehicle or test article were added to 2.0 ml of molten selective top agar at 45$\pm$2°C. When plating the positive controls, the test article aliquot was replaced by a 50 μl aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37$\pm$2°C. Plates that were not counted immediately following the incubation period were stored at 4$\pm$2°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity and precipitate by using a dissecting microscope. This toxicity and precipitate were scored relative to the vehicle control plate, using the criteria and codes that appear in Figure 3. Revertant colonies for a given tester strain and activation condition, except for the positive controls, were counted either entirely by automated colony counter or entirely by hand unless 1) the assay is the dose range finding assay, 2) the plate meets the criteria for toxicity defined in the protocol or 3) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually except as noted above.

For each replicate plating, the mean and standard deviation of the number of revertants per plate was calculated. The results of these calculations are presented on the individual strain data forms.

Criteria for a Valid Test and Evaluation of Results

The following criteria must be met for the mutagenicity assay to be considered valid. All tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*), the deletion in the *uvr*B gene and the characteristic mean number of spontaneous revertants in the vehicle control as shown below:

TA98	10	-	50
TA100	80	-	240
TA1535	5	-	45
TA1537	3	-	21
TA1538	5	-	35

Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels are required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn. Historical vehicle and positive control data are presented in Appendix I.

For the test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test article. Data sets for strains TA1535, TA1537 and TA1538 will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value. Data sets for strains TA98 and TA100 will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Archives

All experimental records (raw data and appropriate reports) of the study are maintained in the Microbiological Associates, Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850. The Director of the Quality Assurance Unit is responsible for maintaining the archives.

Bacterial Background Lawn Evaluation Code
Figure 3

The condition of the background bacterial lawn is evaluated, first macroscopically and then microscopically (using a dissecting microscope). Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn. The evaluation is recorded using the code described below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
SP	Slight Precipitate	Distinguished by noticeable precipitate on the plate, either macro or microscopically; however, any precipitate particles detected by the automated colony counter must total less than 10% of the revertant colony count (e.g., ≤3 particles on a plate with 30 revertants.)
MP	Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate such that the number of precipitate particles detected by the automated colony counter exceeds 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants). This normally requires the plate to be hand counted unless 1) the plate meets the criteria for toxicity defined in the protocol or 2) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count.
HP	Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the revertant colonies difficult to distinguish from the precipitate. This normally requires the plate to be hand counted unless 1) the plate meets the criteria for toxicity defined in the protocol or 2) the plate exhibits definitive mutagenic activity and precipitate accounts for <25 % of the machine-determined revertant count.
Thus, 3MP would indicate a plate observed to have a moderately reduced background lawn with a marked amount of precipitate that required the plate to be counted manually.		

RESULTS AND DISCUSSION

Dose Range-Finding Study

The results of the dose range-finding study are presented in Table 1. The maximum dose tested was 6050 μg per plate. This dose was delivered to the test system as a clear solution in water using a plating aliquot of 50 μl.

The results of the dose range-finding study indicate that no precipitate but toxicity was observed. Therefore, the maximum dose that was plated in the mutagenicity assay was 3333 μg per plate.

Mutagenicity Assay

The results of the mutagenicity assay are presented in Tables 2 through 12 and summarized in Tables 13 and 14. These data were generated in Experiments B1 and B2. No precipitate but toxicity was observed.

In Experiment B1, no positive responses were observed with any of the tester strains in the presence and absence of S9 activation. To clarify the response, tester strain TA100 in the presence of S9 activation was retested in Experiment B2. Clarification was necessary due to the 1.4-fold increase that was observed at 333 μg/plate with definitive toxicity at the next higher dose level, 1000 μg/plate. The doses were adjusted as shown in the data tables to include additional dose levels in the critical portion of the dose-response curve.

In Experiment B2, no positive response was observed with tester strain TA100 in the presence of S9 activation. Despite the adjustment in dose levels, definitive toxicity was again observed at 1000 μg/plate with no appreciable increase (maximum 1.4-fold at 500 μg/plate) in revertants per plate at the lower dose levels. Although a reproducible 1.4-fold increase was observed with tester strain TA100 in the presence of S9 activation, these responses do not meet the criteria to be evaluated as a positive response.

All criteria for a valid study were met as described in the protocol.

CONCLUSION

The results of the *Salmonella* Plate Incorporation Mutagenicity Assay indicate that under the conditions of this study, test article **WR6026 Dihydrochloride** did not cause a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

Salmonella Mutagenicity Assay

Dose Range-Finding Study

Table 1

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501
Experiment No. : A1
Date Plated : 12/14/94
Counted by : machine
Vehicle : water
Plating Aliquot : 50 µl

	TA100			
	With S9 Activation		Without Activation	
Test Article Concentration µg per plate	Revertants per plate	Background Code[a]	Revertants per plate	Background Code[a]
Vehicle	170	1	147	1
8.07	178	1	105	1
12.1	149	1	131	1
40.3	181	1	136	1
80.7	193	1	127	1
121	219	1	132	1
403	214	1	159	1
807	135	1	135	3
1210[b]	34	3	4	4
4033[b]	0	5	0	5
6050[b]	0	5	0	5

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 2

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA98 Cells Seeded : 0.8 X 10^8
Liver Microsomes : None Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	25	1		
	03	15	1	18	6
10	01	15	1		
	02	13	1		
	03	12	1	13	2
33	01	13	1		
	02	15	1		
	03	19	1	16	3
100	01	17	1		
	02	15	1		
	03	20	1	17	3
333	01	8	1		
	02	23	1		
	03	17	1	16	8
1000	01	9	4		
	02	10	3		
	03	12	4	10	2
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	330	1		
	02	337	1		
	03	387	1	351	31

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 3

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA98 Cells Seeded : 0.8 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	26	1		
	02	19	1		
	03	25	1	23	4
10	01	14	1		
	02	19	1		
	03	20	1	18	3
33	01	13	1		
	02	22	1		
	03	24	1	20	6
100	01	25	1		
	02	17	1		
	03	28	1	23	6
333	01	26	1		
	02	20	1		
	03	38	1	28	9
1000	01	18	1		
	02	15	1		
	03	13	1	15	3
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	812	1		
	02	848	1		
	03	843	1	834	20

[a]Background bacteriel evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 4

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA100 Cells Seeded : 1.2 X 10^8
Liver Microsomes : None Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	167	1		
	02	163	1		
	03	129	1	153	21
10	01	178	1		
	02	159	1		
	03	171	1	169	10
33	01	281	1		
	02	258	1		
	03	247	1	262	17
100	01	202	1		
	02	165	1		
	03	169	1	179	20
333	01	201	1		
	02	215	1		
	03	213	1	210	8
1000	01	206	4		
	02	105	4		
	03	137	4	149	52
3333[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control sodium azide 1.0 µg per plate					
	01	908	1		
	02	963	1		
	03	894	1	922	36

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 5

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA100 Cells Seeded : 1.2 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	162	1		
	02	187	1		
	03	203	1	184	21
10	01	176	1		
	02	185	1		
	03	179	1	180	5
33	01	178	1		
	02	186	1		
	03	209	1	191	16
100	01	202	1		
	02	190	1		
	03	195	1	196	6
333	01	250	1		
	02	236	1		
	03	277	1	254	21
1000	01	101	4		
	02	186	3		
	03	151	3	146	43
3333[b]	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	1261	1		
	02	1418	1		
	03	1381	1	1353	82

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Plates were hand counted

Salmonella Mutagenicity Assay

Table 6

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA1535 Cells Seeded : 1.4 X 10^8
Liver Microsomes : None Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	14	1		
	03	15	1	13	2
10	01	24	1		
	02	19	1		
	03	22	1	22	3
33	01	17	1		
	02	10	1		
	03	12	1	13	4
100	01	17	1		
	02	16	1		
	03	10	1	14	4
333	01	22	1		
	02	10	1		
	03	13	1	15	6
1000	01	0	5		
	02	5	3		
	03	7	4	4	4
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control sodium azide 1.0 µg per plate					
	01	758	1		
	02	690	1		
	03	698	1	715	37

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

Salmonella Mutagenicity Assay

Table 7

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA1535 Cells Seeded : 1.4 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	17	1		
	03	20	1	17	4
10	01	16	1		
	02	17	1		
	03	15	1	16	1
33	01	19	1		
	02	15	1		
	03	17	1	17	2
100	01	12	1		
	02	17	1		
	03	20	1	16	4
333	01	9	1		
	02	15	1		
	03	17	1	14	4
1000	01	10	1		
	02	14	1		
	03	18	1	14	4
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	145	1		
	02	137	1		
	03	179	1	154	22

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 8

Test Article Id	: WR6026 Dihydrochloride		
Study Number	: G94BT87.501	Experiment No	: B1
Strain	: TA1537	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: None	Date Plated	: 12/22/94
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	3	1		
	02	4	1		
	03	8	1	5	3
10	01	12	1		
	02	7	1		
	03	8	1	9	3
33	01	4	1		
	02	9	1		
	03	3	1	5	3
100	01	8	1		
	02	9	1		
	03	4	1	7	3
333	01	11	3		
	02	9	3		
	03	6	1	9	3
1000	01	2	4		
	02	2	4		
	03	1	4	2	1
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 9-aminoacridine 75 µg per plate[b]					
	01	710	1		
	02	416	1		
	03	442	1	523	163

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate

[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 9

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA1537 Cells Seeded : 0.7 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	5	1		
	02	4	1		
	03	7	1	5	2
10	01	6	1		
	02	6	1		
	03	3	1	5	2
33	01	6	1		
	02	6	1		
	03	7	1	6	1
100	01	6	1		
	02	9	1		
	03	9	1	8	2
333	01	6	1		
	02	6	1		
	03	7	1	6	1
1000	01	2	1		
	02	6	1		
	03	8	1	5	3
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	99	1		
	02	101	1		
	03	160	1	120	35

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 10

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA1538 Cells Seeded : 0.7 X 10^8
Liver Microsomes : None Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	6	1		
	02	6	1		
	03	14	1	9	5
10	01	2	1		
	02	10	1		
	03	8	1	7	4
33	01	4	1		
	02	7	1		
	03	8	1	6	2
100	01	6	1		
	02	4	1		
	03	7	1	6	2
333	01	7	1		
	02	8	1		
	03	11	1	9	2
1000	01	2	4		
	02	5	4		
	03	3	4	3	2
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-nitrofluorene 1.0 µg per plate[b]					
	01	615	1		
	02	589	1		
	03	514	1	573	52

[a]Background bacterial evaluation code
1=Normal 2=Slightly raducad 3=Moderataly reduced
4=Extremely raduced 5=Absent 6=Obscured by precipitate
SP=Slight pracipitate MP=Moderate precipitate HP=Haavy precipitate
[b]Positiva control platas were machine counted

Salmonella Mutagenicity Assay

Table 11

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1
Strain : TA1538 Cells Seeded : 0.7 X 10^8
Liver Microsomes : Rat liver S9 Date Plated : 12/22/94
Vehicle : water
Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	13	1		
	02	10	1		
	03	14	1	12	2
10	01	10	1		
	02	13	1		
	03	19	1	14	5
33	01	5	1		
	02	3	1		
	03	12	1	7	5
100	01	13	1		
	02	6	1		
	03	7	1	9	4
333	01	8	1		
	02	8	1		
	03	13	1	10	3
1000	01	5	3		
	02	11	2		
	03	7	1	8	3
3333	01	0	5		
	02	0	5		
	03	0	5	0	0
Positive Control 2-aminoanthracene 1.0 µg per plate[b]					
	01	1409	1		
	02	1205	1		
	03	1369	1	1328	108

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
[b]Positive control plates were machine counted

Salmonella Mutagenicity Assay

Table 12

Test Article Id	: WR6026 Dihydrochloride		
Study Number	: G94BT87.501	Experiment No	: B2
Strain	: TA100	Cells Seeded	: 0.7 X 10^8
Liver Microsomes	: Rat liver S9	Date Plated	: 01/04/95
Vehicle	: water		
Plating Aliquot	: 50 µl	Counted by	: machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code[a]	Average Revertants	Standard Deviation
Vehicle	01	153	1		
	02	208	1		
	03	185	1	182	28
10	01	181	1		
	02	193	1		
	03	173	1	182	10
33	01	194	1		
	02	198	1		
	03	194	1	195	2
100	01	226	1		
	02	180	1		
	03	188	1	198	25
200	01	209	1		
	02	214	1		
	03	216	1	213	4
333	01	234	1		
	02	233	1		
	03	218	1	228	9
500	01	282	1		
	02	NC			
	03	228	1	255	38
750	01	120	1		
	02	108	1		
	03	157	1	128	26
1000[b]	01	16	2		
	02	19	2		
	03	12	2	16	4
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	916	1		
	02	815	1		
	03	893	1	875	53

[a]Background bacterial evaluation code
1=Normal 2=Slightly reduced 3=Moderately reduced
4=Extremely reduced 5=Absent 6=Obscured by precipitate
SP=Slight precipitate MP=Moderate precipitate HP=Heavy precipitate
NC=No count due to procedural error in which plate did not receive an aliquot of tester strain
[b]Plates were hand counted

Salmonella Mutagenicity Assay
Summary of Results

Table 13

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (μg)	TA98	TA100	TA1535	TA1537	TA1538
0.0	18 ± 6	153 ± 21	13 ± 2	5 ± 3	9 ± 5
10	13 ± 2	169 ± 10	22 ± 3	9 ± 3	7 ± 4
33	16 ± 3	262 ± 17	13 ± 4	5 ± 3	6 ± 2
100	17 ± 3	179 ± 20	14 ± 4	7 ± 3	6 ± 2
333	16 ± 8	210 ± 8	15 ± 6	9 ± 3	9 ± 2
1000	10 ± 2	149 ± 52	4 ± 4	2 ± 1	3 ± 2
3333	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pos	351 ± 31	922 ± 36	715 ± 37	523 ± 163	573 ± 52

Liver Microsomes: Rat liver S9

Dose (μg)	TA98	TA100	TA1535	TA1537	TA1538
0.0	23 ± 4	184 ± 21	17 ± 4	5 ± 2	12 ± 2
10	18 ± 3	180 ± 5	16 ± 1	5 ± 2	14 ± 5
33	20 ± 6	191 ± 16	17 ± 2	6 ± 1	7 ± 5
100	23 ± 6	196 ± 6	16 ± 4	8 ± 2	9 ± 4
333	28 ± 9	254 ± 21	14 ± 4	6 ± 1	10 ± 3
1000	15 ± 3	146 ± 43	14 ± 4	5 ± 3	8 ± 3
3333	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pos	834 ± 20	1353 ± 82	154 ± 22	120 ± 35	1328 ± 108

0.0 = Vehicle plating aliquot of 50 μl
Pos = Positive Control concentrations as specified in Materials and Methods section.

Salmonella Mutagenicity Assay
Summary of Results

Table 14

Test Article Id : WR6026 Dihydrochloride
Study Number : G94BT87.501 Experiment No : B2

Average Revertants Per Plate ± Standard Deviation
Liver Microsomes: Rat liver S9

Dose (μg)	TA100
0.0	182 ± 28
10	182 ± 10
33	195 ± 2
100	198 ± 25
200	213 ± 4
333	228 ± 9
500	255 ± 38
750	128 ± 26
1000	16 ± 4
Pos	875 ± 53

0.0 = Vehicle plating aliquot of 50 μl
Pos = Positive Control concentrations as specified in Materials and Methods section.

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McCann, J., E. Choi, E. Yamasaki and B.N. Ames (1975) Detection of Carcinogens as Mutagens in the *Salmonella*/Microsome Test: Assay of 300 Chemicals, Proc. Natl. Acad. Sci. USA, 72:5135-5139.

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APPENDIX I

Historical Control Data

Historical Vehicle and Positive Control Values Plate Incorporation Method 1991 - 1993											
S9 Activation	Parameter	TA98		TA100		TA1535		TA1537		TA1538	
		Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos	Veh	Pos
None	Mean	19	240	130	606	11	434	6	651	8	409
	SD	6	135	27	158	4	149	3	666	4	249
	Minimum	3	29	68	251	1	62	1	13	0	24
	Maximum	62	1686	278	1768	40	1862	29	3879	36	2495
Rat	Mean	28	892	160	972	13	105	8	112	13	992
	SD	8	543	32	493	4	127	3	94	5	525
	Minimum	6	26	76	203	0	20	0	11	0	59
	Maximum	62	3099	268	2953	39	2153	25	799	38	2787

Veh = Vehicle Control (including but not limited to deionized water, dimethylsulfoxide, ethanol and acetone)
Pos = Positive Control

APPENDIX II

Study Protocol

APPROVED
CC 12-14-94

MA Study Number: G94BT87.501

SALMONELLA PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

DRAFT

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: Toxicology Research Laboratory

2.2 Address: University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T

2.4 Sponsor Project #: UIC-14 This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratroy, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: WR6026 Dihydrochloride (mole fraction = 0.825)
Bottle No. BK01845

Storage: -15 to -20°C
Tightly closed in a desiccator
Protect from light

3.2 Controls: Negative: Test article solvent

Positive: 9-aminoacridine
2-aminoanthracene
2-nitrofluorene
sodium azide

corrected for omission.
RS 12/13/94

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

4.4 Associate Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12/14/94

5.2 Proposed Experimental Completion Date: 1/11/95

5.3 Proposed Report Date: 1/25/95

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames *et al.* (1975).

Genotype of the *S. typhimurium* Strains Used for Mutagen Testing

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R-factor
TA1535	TA1537	TA1538	*rfa*	Δ*uvr*B	-
TA100		TA98	*rfa*	Δ*uvr*B	+R

Each tester strain contains, in addition to a mutation in the histidine operon, two additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The second mutation is a deletion in the *uvr*B gene resulting in a deficient DNA excision-repair system.

Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test article will be tested at a minimum of five dose levels along with appropriate vehicle and positive controls with tester strains TA98, TA100, TA1535, TA1537 and TA1538 with and without S9 activation. All dose levels of test article, vehicle controls and positive controls will be plated in triplicate.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to: deionized water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, that permits preparation of the highest workable/soluble stock concentration, up to 500 mg/ml.

7.2 Preliminary Toxicity Assay to Select Dose Levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test article assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA100 to vehicle and to at least eight concentrations of test article, one plate per dose level, in the presence and absence of S9 activation. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated both microscopically and macroscopically following the incubation period. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity. In the event that the test article cannot be dissolved at a high enough concentration in an appropriate vehicle to be toxic or if test article precipitate is present on the plates after incubation, the Sponsor will be consulted prior to selection of dose levels.

7.3 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Positive Controls

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1535	+	2-aminoanthracene	1.0
	-	sodium azide	1.0
TA1537	+	2-aminoanthracene	1.0
	-	9-aminoacridine	75
TA1538	+	2-aminoanthracene	1.0
	-	2-nitrofluorene	1.0

7.4.2 Vehicle Controls

Appropriate vehicle controls will be plated for each tester strain with and without S9 activation.

7.4.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced

with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/ml.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.8 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light.

7.9 Treatment of Test System

One-half milliliter (0.5 ml) of S9 mix or Sham mix, 100 µl of tester strain and 50 µl of vehicle, test article dilution or positive control will be added to 2.0 ml of molten selective top agar at 45±2°C. When necessary to achieve the target concentration, aliquots of other than 50 µl of test article/vehicle/positive control will be plated. The mixture will be vortex mixed and overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates

that are not counted immediately following the incubation period will be stored at 4±2°C.

7.10 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed by demonstrating sensitivity to crystal violet. The presence of the *uvr*B mutation will be confirmed by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; TA1538, 5 - 35.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three fold increase over the respective mean vehicle control value for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that fewer than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article as specified below:

9.1 Strains TA1535, TA1537 and TA1538

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value.

9.2 Strains TA98 and TA100

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data. Results presented will include:

- bacterial tester strain description
- test conditions, including dose levels and rationale for selection, number of plates per test point, toxicity, media, type and composition of metabolic activation system, treatment procedures, positive and negative controls.
- individual plate counts
- mean and standard deviation of revertant colonies per plate
- dose response relationship, if applicable

- evaluation of results
- historical control values

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with EPA AND FDA test guidelines. This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor require that samples of the Test Article dosing solutions be returned? YES If so, indicate which doses and volume will be required.

_____high only;
_____high and low only;
___X___all;
volume required ____10 ml (if possible)_.

The dosing solutions will be frozen and sent to UIC for analysis.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research *31*:347-364.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

14.0 APPROVAL

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) — Date 11/1/94

Dr. George Schieferstein (US Army Contracting Officer's Representative) — Date 11/10/94

MA Study Director — Date 12/13/94

APPENDIX 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY REPORT

DRAFT

SECOND REVISED DRAFT REPORT

Study Title

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

Test Article

WR6026 Dihydrochloride

Authors

Richard H. C. San, Ph.D.
Jane J. Clarke, B.A.

Study Completion Date

Pending Final Report

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

Laboratory Study Number

G94BT87.702

Sponsor Project Number

UIC-14

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

STATEMENT OF COMPLIANCE

Microbiological Associates, Inc.'s Study No. G94BT87.702, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

for RS

Richard H. C. San, Ph.D.
Study Director

3/13/95
Date

QUALITY ASSURANCE STATEMENT

DRAFT

Study Title: L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Study Number: G94BT87.702

Study Director: Richard H. C. San, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 16 DEC 94, TO STUDY DIR 16 DEC 94, TO MGMT 16 DEC 94
PHASE: PROTOCOL REVIEW

INSPECT ON 06 JAN 95, TO STUDY DIR 09 JAN 95, TO MGMT 09 JAN 95
PHASE: Cell resuspension and addition to the TFT cloning flasks

INSPECT ON 20 JAN 95, TO STUDY DIR 20 JAN 95, TO MGMT 30 JAN 95
PHASE: Draft Report

INSPECT ON 03 MAR 95, TO STUDY DIR 03 MAR 95, TO MGMT 03 MAR 95
PHASE: Draft to Revised Draft Report

INSPECT ON 14 MAR 95-15 MAR 95, TO STUDY DIR 15 MAR 95, TO MGMT 15 MAR 95
PHASE: Revised Draft to 2nd Revised Draft Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

DBM 3-15-95

Diane B. Madsen
QUALITY ASSURANCE

DATE

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

SECOND REVISED DRAFT REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR6026 Dihydrochloride**

Test Article Bottle No.: **Bottle No. BK01845**

Test Article Purity: **>99.4% (provided by Sponsor)**

Test Article Mole Fraction: **0.825 (provided by Sponsor)**

Sponsor Project No.: **UIC-14**

MA Study No.: **G94BT87.702**

Test Article Description: **light yellow powder**

Storage Conditions: **-20±5°C; protected from exposure to light and moisture**

Test Article Receipt: **December 9, 1994**

Study Initiation: **December 15, 1994**

Laboratory Manager: Jane J. Clarke, B.A.

Study Director: for RCS 3/13/95
Richard H. C. San, Ph.D. Date

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SUMMARY

The test article, WR6026 Dihydrochloride, was tested in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor induced rat liver S9. The assay was performed in two phases. The first phase, the preliminary toxicity assay, was used to establish the dose range for the mutagenesis assay. The second phase, the mutagenesis assay , was used to evaluate the mutagenic potential of the test article. The dosing solutions were adjusted to compensate for the base mole fraction of the test article.

Sterile distilled water was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in sterile distilled water at a maximum concentration of approximately 500 mg base/ml.

In the preliminary toxicity assay, the maximum concentration of WR6026 Dihydrochloride tested was 5000 μg base/ml. Treatment medium was cloudy but with no visible precipitate at concentrations of $\geq$ 1000 μg base/ml. Concentrations of $\leq$ 500 μg base/ml were soluble in treatment medium. Selection of dose levels for the mutation assay was based on reduction of suspension growth relative to the solvent control. Substantial toxicity, i.e., suspension growth of $\leq$50% of the solvent control, was observed at 50 μg base/ml for the non-activated cultures and 0.5 μg base/ml for the S9-activated cultures. Based on these findings, the doses chosen for the mutagenesis assay ranged from 1.0 to 50 μg base/ml for the non-activated cultures and from 0.1 to 5.0 μg base/ml for the S9-activated cultures.

In the mutagenesis assay, no positive responses, i.e., treated cultures with mutant frequencies at least twice that of the solvent control, were observed. No visible precipitate was observed in treatment medium at any concentration. Based on suspension growth relative to the solvent controls, non-activated cultures treated with 1.0 to 20 μg base/ml and S9-activated cultures treated with 0.2 to 0.75 μg base/ml were cloned. Toxicity, i.e., total growth of $\leq$50% of the solvent control, was observed at doses of 20 μg base/ml in the non-activated cultures and 0.5 μg base/ml in the S9-activated cultures.

Under the conditions of this study, test article WR6026 Dihydrochloride was concluded to be negative in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR6026 Dihydrochloride, was received by Microbiological Associates, Inc. on December 9, 1994 and was assigned the code number 94BT87. The test article was characterized by the Sponsor as a light yellow powder, which should be stored at -15 to -20°C, protected from light, tightly closed, with desiccant. Its purity was given as >99.4%. Upon receipt, the test article was described as a light yellow powder and was stored at -20±5°C, protected from exposure to light and moisture.

The vehicle used to deliver WR6026 Dihydrochloride was sterile distilled water (CAS 7732-18-5) obtained from Gibco. The dosing solutions were adjusted to compensate for the base mole fraction of the test article. Aliquots of dosing solution preparations were retained by MA to be shipped to the Sponsor for chemical analysis upon completion of the study.

Ethyl methanesulfonate (EMS), CAS 62-50-0, was obtained from Eastman Kodak Chemical Company and was diluted to stock concentrations of 25 and 50 µl/ml for use as the positive control for the non-activated test system. 7,12-Dimethylbenz(a)-anthracene (7,12-DMBA), CAS 57-97-6, was obtained from Eastman Kodak Chemical Company and was diluted to stock concentrations of 250 and 500 µg/ml for use as the positive control for the S9-activated test system.

MATERIALS AND METHODS

Test System

L5178Y cells, clone 3.7.2C, were obtained from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, NC. (Clive and Spector, 1975). Each lot of cryopreserved cells was tested using the agar culture and Hoechst staining procedures and found to be free of mycoplasma contamination. Prior to use in the assay, L5178Y cells were cleansed of spontaneous $TK^{-/-}$ cells by culturing in a restrictive medium (Clive and Spector, 1975).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor-1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤ -70°C until used. Each bulk preparation of S9 was assayed

for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was mixed with the cofactors and Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F_0P) to contain 250 μl S9, 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), 11.25 mg DL-isocitric acid and 750 μl F_0P/ml S9-activation mixture and kept on ice until used. The cofactor/F_0P mixture was filter sterilized and adjusted to pH 7.0 prior to the addition of S9.

Solubility Test

A solubility test was conducted to select the vehicle. When the test was conducted using distilled water the test article was found to be soluble at 500 mg/ml. Therefore, the vehicle of choice was distilled water because it permitted preparation of a stock concentration of 500 mg/ml.

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the optimal dose levels for the mutagenesis assay. L5178Y cells were exposed to the vehicle alone and nine concentrations of test article ranging from 0.5 to 5000 μg base/ml in both the absence and presence of S9-activation.

Cell population density was determined 24 and 48 hours after the initial exposure to the test article. The cultures were adjusted to $3x10^5$ cells/ml after 24 hours only. Cultures with less than $3x10^5$ cells/ml were not adjusted. Toxicity was measured as suspension growth relative to the growth of the solvent controls.

Mutagenesis Assay

The mutagenesis assay was used to evaluate the mutagenic potential of the test article. L5178Y mouse lymphoma cells were exposed to the vehicle alone and eight concentrations of test article in duplicate in both the absence and presence of S9. Positive controls, with and without S9-activation, were tested concurrently.

Treatment of the Target Cells

The mutagenesis assay was performed according to a protocol described by Clive and Spector (1975). Treatment was carried out in conical tubes by combining 6×10^6 L5178Y/$TK^{+/-}$ cells and 100 μl dosing solution of test or control article in solvent or solvent alone in a total volume of 10 ml F_0P medium or S-9 activation mixture. A total of eight concentrations of test article were tested in duplicate. Two control tubes received solvent only and the positive controls were treated with EMS (at final concentrations of 0.25 and 0.5 μl/ml) and 7,12-DMBA (at final concentrations of 2.5 and 5.0 μg/ml). Treatment tubes were gassed with $5 \pm 1\%$ $C0_2$ in air, capped tightly, and incubated with mechanical mixing for 4 hours at $37 \pm 1°C$. The preparation and addition of the test article dosing solutions were carried out under amber lighting and

the cells were incubated in the dark during the 4-hour exposure period. After the treatment period of 4 hours at 37±1°C, the cells were washed twice with F_0P supplemented with 10% horse serum and 4 Mm L-glutamine ($F_{10}P$). After the second wash, the cells were resuspended in $F_{10}P$, gassed with 5±1% CO_2 in air and placed on the roller drum apparatus at 37±1°C.

Expression of the Mutant Phenotype

For expression of the mutant phenotype, the cultures were counted using an electronic cell counter and adjusted to $3x10^5$ cells/ml at approximately 24 and 48 hours after treatment in 20 and 10 ml total volume, respectively. Cultures with less than $3x10^5$ cells/ml were not adjusted.

For expression of the $TK^{-/-}$ cells, cells were placed in cloning medium (C.M.) containing 0.23% granulated agar. Two flasks per culture to be cloned were labeled with the test article concentration, activation condition, and either TFT (trifluorothymidine, the selective agent) or V.C. (viable count). Each flask was prewarmed to 37±1°C, filled with 100 ml C.M., and placed in an incubator shaker at 37±1°C until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were then diluted in C.M. to concentrations of $3x10^6$ cells/100 ml C.M. for the TFT flask and 600 cells/100 ml C.M. for the VC flask. After the dilution, 1.0 ml of stock solution of TFT was added to the TFT flask (final concentration of 3 μg/ml) and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37±1°C. After 15 minutes, the flasks were removed one at a time, and 33 ml of the cell suspension was pipetted into each of three appropriately labeled petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 20 minutes. The plates were then incubated at 37±1°C in a humidified 5±1% CO_2 atmosphere for 10-12 days.

Scoring Procedures

After the incubation period, both the TFT plates and the V.C. plates were counted for the total number of colonies per plate. Three counts per plate were made on an automatic colony counter and the median count was recorded. In the case of a positive response, the diameters of the TFT colonies were determined over a range of 0.2 to 1.1 mm. The rationale for this procedure is as follows: Mutant L5178Y TK-/- colonies exhibit a characteristic frequency distribution of colony sizes. The precise distribution of large and small TFT-resistant mutant colonies appears to be the characteristic mutagenic "finger-print" of carcinogens in the L5178Y TK+/- system (Clive *et al.*, 1979; DeMarini *et al.*, 1989). Clive and co-workers (1979) and Hozier *et al.* (1981) have presented evidence to substantiate the hypothesis that the small colony variants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Kozak and Ruddle, 1977). They suggested that large colony mutants received very localized damage, possibly in the form of a point mutation or small deletion within the TK locus, while small colony mutants received damage to collateral loci concordant with the loss of TK activity.

Evaluation of Results

The mutant frequency (number of mutants per 10^6 surviving cells) was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C. plates and multiplying by the dilution factor ($2x10^{-4}$).

In evaluation of the data, increases in mutant frequencies which occurred only at highly toxic concentrations (i.e., less than 10% total growth) were not considered biologically relevant. All conclusions were based on sound scientific judgement; however, as a guide to interpretation of the data, the test article was considered to induce a positive response if a concentration-related increase in mutant frequency was observed and more than one dose level with 10% or greater total growth exhibited a mutant frequency two-fold greater than the solvent control. A doubling above background at one or more dose levels with 10% or greater total growth with no evidence of a dose-response was considered equivocal. Test articles not producing a doubling above background at one or more dose levels with 10% or greater total growth were concluded to be negative.

Criteria for a Valid Test

The following criteria must be met for the mutagenesis assay to be considered valid. The mutant frequency of the positive controls must be at least twice that of the appropriate solvent control cultures. The spontaneous mutant frequency of the solvent controls must be between 20 and 100 per 10^6 surviving cells. The cloning efficiency of the solvent controls must be greater than 50%.

Archives

Upon completion of the final report, all raw data and reports are maintained in Microbiological Associates, Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Solubility Test

Sterile distilled water was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in sterile distilled water at a maximum concentration of approximately 500 mg base/ml.

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in Table 1. The maximum dose tested in the preliminary toxicity assay was 5000 μg base/ml. No test article precipitate was observed at any dose level in treatment medium. The osmolality of the solvent control was 302 mOsm/kg and the osmolality of the top dose, 5000 μg base/ml,

was 304 mOsm/kg. Suspension growth relative to the solvent controls was 63% at 10 μg base/ml without activation and 6% at 1.0 μg base/ml with S9 activation. Based on the results of the toxicity test, the doses chosen for the mutagenesis assay ranged from 1.0 to 50 μg base/ml for the non-activated cultures and 0.1 to 5.0 μg base/ml for the S9-activated cultures.

Mutagenesis Assay

The results of the mutagenesis assay are presented in Tables 2 through 5. Based on suspension growth relative to the solvent controls, non-activated cultures treated with concentrations of 1.0, 5.0, 10, 15 and 20 μg base/ml were cloned and produced a range in suspension growth of 103% to 31%. Based on suspension growth relative to the solvent controls, S9-activated cultures treated with concentrations of 0.2, 0.3, 0.4, 0.5 and 0.75 μg base/ml were cloned and produced a range in suspension growth of 98% to 10%. No test article precipitate was observed at any dose level in treatment medium.

No cultures exhibited a mutant frequency which was at least two times the mean mutant frequency of the solvent controls. The total growths ranged from 100% to 24% for the non-activated cultures at concentrations of 1.0 to 20 μg base/ml and 97% to 5% for the S9-activated cultures at concentrations of 0.2 to 0.75 μg base/ml. A response trend was not observed in the non-activated or S9-activated systems.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay indicate that under the conditions of this study, WR6026 Dihydrochloride did not cause a positive response in the non-activated and S9-activated systems and was concluded to be negative.

REFERENCES

Clive D., Johnson K.O., Spector J.F.S., Batson A.G. and Brown M.M.M. (1979) Validation and characterization of the L5178Y TK+/- mouse lymphoma mutagen assay system. Mutation Res. 59:61-108.

Clive, D. and Spector, J.F.S. (1975) Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178 TK+/- mouse lymphoma cells. Mutation Res. 31:17-29.

DeMarini, D.M., Brockman, H.E., de Serres, F.J., Evans, H.H., Stankowski Jr, L.F. and Hsie, A.W. (1989) Specific-locus mutations induced in eukaryotes (especially mammalian cells) by radiation and chemicals: a perspective. Mutation Res. 220:11-29.

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Kozak, C.A. and Ruddle, F.H. (1977) Assignment of the genes for thymidine kinase and galactokinase to *Mus musculus* chromosome 11 and the preferential segregation of this chromosome in Chinese hamster/mouse somatic cell hybrids. Somatic Cell Genet. 3:121-133.

TABLE 1

PRELIMINARY TOXICITY TEST USING WR6026 Dihydrochloride

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	% of Control[c]
WITHOUT ACTIVATION				
Water A	1.329	1.494	22.1	
Water B	1.377	1.541	23.6	
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:				
0.5	1.487	1.378	22.8	100
1.0	1.413	1.385	21.7	95
5.0	1.243	1.130	15.6	68
10	1.025	1.273	14.5	63
50	0.011	0.010	0.0	0
100	0.009	0.016	0.0	0
500	0.043	0.031	0.0	0
1000	0.017	0.010	0.0	0
5000	0.013	0.019	0.0	0
WITH S9 ACTIVATION				
Water A	0.999	1.534	17.0	
Water B	0.970	1.581	17.0	
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:				
0.5	0.467	0.755	3.9	23
1.0	0.307	0.329	1.1	6
5.0	0.068	0.041	0.0	0
10	0.047	0.031	0.0	0
50	0.029	0.012	0.0	0
100	0.010	0.010	0.0	0
500	0.125	0.095	0.0	0
1000	0.046	0.038	0.0	0
5000	0.015	0.047	0.0	0

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

TABLE 2

CLONING DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH WR6026 Dihydrochloride IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)		Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
Water	A	25/3	± 5	156/3	± 6	32		
Water	B	20/3	± 2	155/3	± 2	26		
Mean Solvent Mutant Frequency= 29								
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:								
1.0	A	18/3	± 2	155/3	± 9	23	-6	96
	B	20/3	± 3	143/3	± 3	28	-1	95
5.0	A	23/3	± 3	150/3	± 11	31	2	84
	B	20/3	± 4	167/3	± 15	24	-5	100
10	A	15/3	± 3	142/3	± 9	21	-8	75
	B	20/3	± 4	153/3	± 12	26	-3	77
15	A	10/3	± 3	145/3	± 6	14	-15	58
	B	19/3	± 4	136/3	± 6	28	-1	50
20	A	20/3	± 7	139/3	± 7	29	0	28
	B	18/3	± 2	113/3	± 6	32	3	24
Positive Control - Ethyl Methanesulfonate (µl/ml)								
DMSO	A	13/3	± 1	156/3	± 2	17		
DMSO	B	20/3	± 2	146/3	± 2	27		
Mean Solvent Mutant Frequency= 22								
0.25		271/3	±14	116/3	± 6	467	445	56
0.50		358/3	± 8	90/3	± 10	796	774	31

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = (Average # TFT colonies / average # VC colonies) x 200

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 3

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS
TREATED WITH WR6026 Dihydrochloride
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)		Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
Water	A	1.440	1.559	24.9		156		
Water	B	1.613	1.454	26.1		155		
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:								
1.0	A	1.399	1.578	24.5	96	155	100	96
	B	1.512	1.567	26.3	103	143	92	95
5.0	A	1.385	1.463	22.5	88	150	96	84
	B	1.401	1.523	23.7	93	167	107	100
10	A	1.303	1.435	20.8	82	142	91	75
	B	1.260	1.433	20.1	79	153	98	77
15	A	1.012	1.394	15.7	62	145	93	58
	B	1.007	1.325	14.8	58	126	87	50
20	A	0.556	1.256	7.8	31	139	89	28
	B	0.553	1.346	8.3	33	113	73	24
30	A	0.098	0.422	1.4	5	++		
	B	0.111	0.488	1.6	6	++		
Positive Control - Ethyl Methanesulfonate (µl/ml)								
DMSO	A	1.554	1.542	26.6		156		
DMSO	B	1.505	1.518	25.4		146		
	0.25	1.166	1.476	19.1	73	116	77	56
	0.50	1.008	1.186	13.3	51	90	60	31

++ - Too toxic to clone

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 4

CLONING DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS
TREATED WITH WR6026 Dihydrochloride
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)		Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
Water	A	22/3	± 3	126/3	± 15	35		
Water	B	29/3	± 4	152/3	± 10	38		
Mean Solvent Mutant Frequency= 37								
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:								
0.2	A	30/3	± 2	129/3	± 21	47	10	86
	B	34/3	± 4	137/3	± 4	50	13	97
0.3	A	27/3	± 4	129/3	± 5	42	5	75
	B	32/3	± 4	151/3	± 11	42	5	94
0.4	A	35/3	± 4	137/3	± 13	51	14	71
	B	33/3	± 5	141/3	± 14	47	10	75
0.5	A	36/3	± 7	106/3	± 5	68	31	34
	B	34/3	± 2	145/3	± 6	47	10	45
0.75	A	31/3	± 4	69/3	± 4	90	53	5
	B	22/3	± 3	73/3	± 8	60	23	5
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)								
DMSO	A	29/3	± 8	128/3	± 3	45		
DMSO	B	35/3	± 1	146/3	± 14	48		
Mean Solvent Mutant Frequency= 46								
2.5		122/3	±13	86/3	± 5	284	238	37
5.0		9/3	± 3	5/3	± 1	360	314	0

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = (Average # TFT colonies / average # VC colonies) x 200

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 5

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS
TREATED WITH WR6026 Dihydrochloride
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
Water A	1.091	1.424	17.3		126		
Water B	1.084	1.679	20.2		152		
WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845:							
0.2 A	1.013	1.553	17.5	93	129	93	86
B	1.085	1.529	18.4	98	137	99	97
0.3 A	0.934	1.479	15.3	81	129	93	75
B	1.014	1.428	16.1	86	151	109	94
0.4 A	0.813	1.508	13.6	72	137	99	71
B	0.829	1.520	14.0	74	141	101	75
0.5 A	0.587	1.287	8.4	45	106	76	34
B	0.621	1.155	8.0	43	145	104	45
0.75 A	0.388	0.440	1.9	10	69	50	5
B	0.391	0.438	1.9	10	73	53	5
1.0 A	0.347	0.300	1.2	6	++		
B	0.353	0.306	1.2	6	++		
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)							
DMSO A	0.982	1.512	16.5		128		
DMSO B	1.026	1.454	16.6		146		
2.5	0.682	1.261	9.6	58	86	63	37
5.0	0.366	0.306	1.2	7	5	4	0

++ - Too toxic to clone

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

APPENDIX I

Historical Control Data

Mouse Lymphoma Historical Control Data

1991-1993

	Non-activated			S9-activated		
	Solvent	0.5μl/ml EMS	0.25μl/ml EMS	Solvent	5.0μg/ml DMBA	2.5μg/ml DMBA
Mean MF	39.1	848.6	447.3	46.9	592.1	236.4
SD	12.3	173.8	73.5	12.6	216.2	61.6
Maximum	80.0	1314.0	691.0	87.0	1358.0	517.0
Minimum	22.0	458.0	294.0	22.0	167.0	100.0

Solvent = Solvent control (Fischer's medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor)
EMS = Ethylmethanesulfonate
DMBA = Dimethylbenz(a)anthracene
MF = Mutant frequency per 10^6 clonable cells
SD = Standard deviation

APPENDIX II

Study Protocol

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

DRAFT

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.0 SPONSOR

2.1 Name: Toxicology Research Laboratory

2.2 Address: University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T

2.4 Sponsor Project #: UIC-14 This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratroy, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: WR6026 Dihydrochloride (mole fraction = 0.825)
Bottle No. BK01845

Storage: -15 to -20°C
Tightly closed in a desiccator
Protect from light

3.2 Controls: Negative: Test article solvent
Positive: Ethyl methanesulfonate (EMS)
7,12-dimethylbenz(a)anthracene (DMBA)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

DRAFT

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic and Cellular Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12/20/94

5.2 Proposed Experimental Completion Date: 1/30/95

5.3 Proposed Report Date: 2/3/95

6.0 TEST SYSTEM

L5178Y/TK$^{+/-}$ mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK$^{+/-}$, clone 3.7.2C, were received from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination. This system has been demonstrated to be sensitive to the mutagenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutation assay will be performed by exposing duplicate cultures of L5178Y/TK$^{+/-}$ cells to a minimum of eight concentrations of test article as well as positive and negative (solvent) controls. Exposures will be for 4 hours in the presence and absence of an S-9 activation system. Following a two day expression period, with daily cell population adjustments, cultures demonstrating 0% to 90% growth inhibition will be cloned, in triplicate, in restrictive medium containing soft agar to select for the mutant phenotype. After a 10 to 12 day selection period, mutant colonies will be enumerated. The mutagenic potential of the test article will be measured by its ability to induce TK$^{+/-}$ → TK$^{-/-}$ mutations. For those test articles demonstrating a positive response, mutant colonies will be sized as an indication of mechanism of action.

7.1 Selection of solvent

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to measure the maximum soluble concentration in a variety of solvents. Solvents compatible with this test system, in order of preference, include, but are not limited to, culture medium

or distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone CAS 67-64-1). The solvent of choice will be that solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

In the preliminary toxicity test, L5178Y/TK$^{+/-}$ cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium but not to exceed 5000 μg/ml. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. After a 4-hour treatment in the presence and absence of S-9 activation, cells will be washed twice with $F_{10}P$ (Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics, supplemented with 10% horse serum and 4mM L-glutamine) and cultured in suspension for two days post-treatment, with cell concentration adjustment on the first day.

Selection of dose levels for the mutation assay will be based on reduction of suspension growth after treatment in the preliminary toxicity test. Unless specified otherwise by the Sponsor, the high dose for the mutation assay will be that concentration exhibiting approximately 100% growth inhibition. The low dose will be selected to exhibit 0% growth inhibition. In the event that the test article cannot be dissolved in solvent or in treatment medium at a high enough concentration to be toxic, the Sponsor will be consulted prior to dose selection.

7.3 Route and Frequency of Administration

Cell cultures will be treated for 4 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The source of S-9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid, 6 mg NADP, and 0.25 ml S-9 homogenate per ml in Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics (F_0P). The S-9 mix will be adjusted to pH 7.

7.5 Controls

7.5.1 Solvent Control

The solvent for the test article will be used as the solvent control.

7.5.2 Positive Controls

Ethyl methanesulfonate (EMS) will be used at two concentrations within the range of 0.2 to 0.6 μl/ml as the positive control for the nonactivated test system. For the S-9 activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two concentrations within the range of 2 to 6 μg/ml.

7.6 Preparation of Target Cells

Prior to use in the assay, L5178Y/TK$^{+/-}$ cells will be cleansed to reduce the frequency of spontaneously occurring TK$^{-/-}$ cells. Using the procedure described by Clive (1975), L5178Y cells will be cultured for 24 hours in the presence of thymidine, hypoxanthine, methotrexate and glycine to poison the TK$^{-/-}$ cells.

L5178Y/TK$^{+/-}$ cells will be prepared at 1 x 10^6 cells/ml in 50% conditioned $F_{10}P$ and 50% F_0P. If cultures are to be treated with more than 100 μl of test article dosing solution, the cell concentration may be adjusted.

7.7 Identification of the Test System

Using a permanent marking pen, the treatment flasks will be identified by the study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in conical tubes by combining 6 x 10^6 L5178Y/TK$^{+/-}$ cells and 100 μl dosing solution of test or control article in solvent or solvent alone in a total volume of 10 ml F_0P medium or S-9 activation mixture. A total of eight concentrations of test article will be tested in duplicate. All pH adjustments will be performed prior to adding S-9 or target cells to the treatment medium. Volumes of test article dosing solution in excess of 100 μl may be used if required to achieve the target final concentration in treatment medium. Treatment tubes will be gassed with 5 ± 1% $C0_2$ in air, capped tightly, and incubated with mechanical mixing for 4 hours at 37 ± 1°C. The preparation and addition of the test article dosing solutions will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

7.9 Expression of the Mutant Phenotype

At the end of the exposure period, the cells will be washed twice with F_0P or $F_{10}P$ and collected by centrifugation. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5 ± 1% CO_2 in air and cultured in suspension at 37 ± 1°C for two days following treatment. Cell population adjustments to 0.3 x 10^6 cells/ml will be made at 24 and 48 hours.

7.10 Selection of the Mutant Phenotype

For selection of the trifluorothymidine (TFT)-resistant phenotype, cells from up to ten treatment conditions demonstrating from 0% to 90% suspension growth inhibition will be plated into three replicate dishes at a density of 1 x 10^4 cells/100mm dish in cloning medium ($F_{10}P$ with 0.23% agar) containing 2-4 μg TFT/ml. For estimation of cloning efficiency at the time of selection, 200 cells/100mm dish will be plated in triplicate in cloning medium free of TFT (viable cell (VC) plates). Plates will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 for 10-12 days.

The total number of colonies per plate will be determined for both the TFT-plates and the VC plates. Three counts per plate will be made using an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the colonies will be counted manually. In the event the test article demonstrates a significant induction in mutant frequency as compared to the solvent controls, the diameters of the TFT colonies will be determined over a range of 0.2 to 1.1 mm.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The spontaneous mutant frequency of the solvent control cultures must be within 20 to 100 TFT-resistant mutants per 10^6 surviving cells. The cloning efficiency of the solvent control group must be greater than 50%.

8.2 Positive Controls

The mutant frequency of the positive controls must be at least twice that of the solvent control.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT-plates by the mean number of

colonies on the VC-plates and multiplying by the dilution factor (2×10^{-4}), and is expressed as TFT-resistant mutants per 10^6 surviving cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations (ie, less than 10% total growth) are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if a concentration-related increase in mutant frequency is observed and more than one dose level with 10% or greater total growth exhibits a mutant frequency two-fold greater than the solvent control. A doubling above background at one or more dose levels with 10% or greater total growth with no evidence of a dose-response will be considered equivocal. Test articles not producing a doubling above background at one or more dose levels with 10% or greater total growth will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

Results presented will include, but not be limited to:

- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant cells
- dose-response relationship, if applicable
- distribution of the mutant colony diameter for each significantly elevated dose level of test article, solvent and positive control (only when the test article induces a positive response)
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc. in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE DRAFT

This protocol was written according to the OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), April, 1984; and the EPA Health Effects Testing Guidelines, Subpart 798.5300 (Detection of Gene Mutations in Somatic Cells in Culture) Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. A confirmatory assay will be required for full OECD and EPA guideline compliance.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes . If so, indicate which doses and volume will be required.

_____high only;

_____high and low only;

__X__all;

volume required 10 ml (if possible) .

The dosing solutions will be frozen and sent to UIC for analysis.

13.0 REFERENCES

Clive, D. and Spector, J.F.S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31:17-29, 1975.

14.0 APPROVAL

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) — Date 11/1/94

Dr. George Schieferstein (US Army Contracting Officer's Representative) — Date 11/10/94.

MA Study Director — Date 12/15/94

APPENDIX 3

CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS REPORT

DRAFT REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

WR6026 Dihydrochloride

Authors

Patrick T. Curry, Ph.D.
Elizabeth Schadly, B.S.

Study Completion Date

(Pending final report)

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, Maryland 20850

Laboratory Study Number

G94BT87.330

Sponsor Project Number

UIC-14

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

STATEMENT OF COMPLIANCE

The cytogenetics study, Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells, G94BT87.330, was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

_____ _____

Patrick T. Curry, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: G94BT87.330

Study Director: Patrick T. Curry, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 14 DEC 94, TO STUDY DIR 14 DEC 94, TO MGMT 14 DEC 94
PHASE: PROTOCOL REVIEW

INSPECT ON 20 DEC 94, TO STUDY DIR 27 DEC 94, TO MGMT 27 DEC 94
PHASE: REMOVAL OF TREATMENT MEDIUM AND REFEEDING OF ACTIVATED SYSTEM

INSPECT ON 21 MAR 95-22 MAR 95, TO STUDY DIR 22 MAR 95, TO MGMT 23 MAR 95
PHASE: Draft Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

CC — March 23, 1995

Claire L. Courtemanche
QUALITY ASSURANCE — DATE

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

DRAFT REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR6026 Dihydrochloride**

Test Article Lot No.: **Bottle No. BK01845**

Test Article Purity: **>99.4% (Provided by Sponsor)**

Test Article Base Mole Fraction: **0.825 (provided by Sponsor)**

MA Study No.: **G94BT87.330**

Test Article Description: **Light yellow powder**

Storage Conditions: **-20°C±5°C, protected from light**

Test Article Receipt: **December 9, 1994**

Study Initiation: **December 13, 1994**

Laboratory Supervisor: **Elizabeth H. Schadly, B.S.**

Associate Study Director: **Donald L. Putman, Ph.D.**

Study Director: PTC
Patrick T. Curry. Ph.D. Date

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SUMMARY

The test article, WR6026 Dihydrochloride, was tested in the chromosome aberration assay using Chinese hamster ovary cells. A preliminary cytotoxicity test was conducted in the absence and presence of an Aroclor-induced S-9 activation system to establish a dose range for testing and to select optimal cell harvest times in the chromosome aberration assay. In this test, the percent reduction in mitotic activity, relative to the solvent control, was used to estimate cytotoxicity; and the average generation time was used to select the optimal cell harvest time. The solvent used for the test article was sterile distilled water. The highest test article concentration assayed in the cytotoxicity test was 5000 μg base/ml. The dosing solutions were adjusted to compensate for the mole fraction (0.825 free base) of the test article. To achieve at least a 50% reduction in mitotic activity, 60 μg base/ml and 6 μg base/ml were selected as the highest dose levels for testing in the nonactivated and S-9 activated portions of the chromosome aberration assay, respectively.

The chromosome aberration assay was conducted at dose levels of 2, 4, 8, 15, 30, and 60 μg base/ml in the absence of metabolic activation; and at 0.4, 0.8, 1.5, 3, and 6 μg base/ml in the presence of an Aroclor-induced S-9 activation system. The dosing solutions were adjusted to compensate for the mole fraction (0.825 free base) of the test article. Due to extensive cell cycle delay observed in the toxicity studies, metaphase cells were collected at 24 hours after initiation of treatment in order to assure microscopic evaluation of first-division metaphase cells. The test article was soluble in solvent (sterile distilled water) at a stock concentration of 6 mg base/ml in the nonactivated test system and at a stock concentration of 0.6 mg base/ml in the S-9 activated test system. The test article was soluble in treatment medium at all concentrations tested. Toxicity, as measured by mitotic inhibition, was approximately 97% and 89% at the highest dose levels evaluated for chromosome aberrations, 30 μg base/ml and 6 μg base/ml, in the nonactivated and S9-activated studies, respectively. No statistically significant increase in chromosome aberrations was observed in the test article treated groups relative to the solvent control group in the nonactivated test system ($p > 0.05$, Fisher's exact test). A statistically significant increase in structural aberrations was observed at the 3 μg base/ml dose level in the S-9 activated test system relative to the solvent control ($p \leq 0.05$, Fisher's exact test). However, the Cochran-Armitage test was negative for a dose trend and no statistically significant increase in the percentage of cells with structural aberrations was observed at this dose level relative to the untreated control ($p > 0.05$, Fisher's exact test). In addition, the percentage of aberrant cells seen at this dose level was within the range of the historical solvent control. Therefore, this observation was not considered to be biologically significant. WR6026 Dihydrochloride was concluded to be negative in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR6026 Dihydrochloride, was received by Microbiological Associates, Inc. on December 9, 1994 and was assigned the code number 94BT87. The test article was characterized by the Sponsor as a light yellow powder that should be stored at -20° to -15°C, protected from light. No expiration date was provided.

Upon receipt, the test article was described as a light yellow powder and was stored at -20°C±5°C, protected from exposure to light. At the time of use, the test article was dissolved in sterile distilled water (CAS # 7732-18-5), obtained from GIBCO. The dosing solutions were adjusted to compensate for the mole fraction (0.825 free base) of the test article.

Mitomycin C (MMC) was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 μg/ml. Cyclophosphamide (CP) was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1 and 2 mg/ml.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981). In order to assure the karyotypic stability of the cell line, cells were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination.

Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of

S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μl S-9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine).

Preliminary Toxicity Assay

The toxicity test was performed for the purpose of selecting dose levels and harvest times for the chromosome aberration assay and consisted of an evaluation of the test article effect on mitotic indices and cell cycle delay. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine) for the nonactivated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl dosing solution of test article in solvent or solvent alone. The cells were treated for 6 hours in the nonactivated test system and for 4 hours in the S-9 activated system. Two hours after initiation of treatment, a 50 μl aliquot of 1mM 5-bromo-2'-deoxyuridine (BrdU) was added to each flask and incubation continued as required. At completion of the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours of BrdU treatment. Two hours prior to harvest by trypsinization, Colcemid® was added to each flask at a final concentration of 0.1 μg/ml. Metaphase preparations were prepared and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides were evaluated for the percentage of first, second and third-plus-subsequent-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The average generation time (AGT) was calculated for each treatment condition and was used to adjust the post-treatment cell harvest times in the chromosome aberration assay. The mitotic index was determined for each treatment condition as the percentage of mitotic cells in a population of 500 cells scored.

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive, untreated, and negative controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37$\pm$1°C in a humidified atmosphere of 5$\pm$1% CO_2 in air for 16-24

hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium for the nonactivated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture was also included.

In the nonactivated study, the cells were exposed continuously for 24 hours at 37 ± 1°C in a humidified atmosphere of $5\pm1\%$ CO_2 in air. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 μg/ml and the flasks returned to the incubator until cell collection.

In the S-9 activated study, the cells were exposed for 4 hours at 37 ± 1°C in a humidified atmosphere of $5\pm1\%$ CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator for an additional 18 hours. At this time, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 μg/ml and the flasks incubated for an additional two hours.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the nonactivated and S-9 activated studies by trypsinization. Cells were collected approximately 24 hours after initiation of treatment. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant fluid decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 ± 2 centromeres were examined under oil

immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads (50 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ($\geq$10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted.

Controls

MMC was used as the positive control in the nonactivated study at final concentrations of 0.08 and 0.15 μg/ml. CP was used as the positive control in the S-9 activated study at final concentrations of 10 and 20 μg/ml. For both positive controls, a dose with sufficient scorable metaphase cells was selected for analysis. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S-9 reaction mixture was used in the untreated control.

Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The AGT was calculated for each treatment condition in the toxicity study as: AGT = (24 hours x 100)/[(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The number and types of aberrations found, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) was calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on a sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive

response when the percentages of cells with aberrations was increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered equivocal. Test articles not demonstrating a statistically significant increase in aberrations were concluded to be negative.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

All raw data, draft and final reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Dose levels for the chromosome aberration assay were selected following preliminary cytotoxicity tests based upon reduction in mitotic index after treatment relative to the solvent control. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.5 μg base/ml to 5000 μg base/ml in the absence and presence of an S-9 reaction mixture. The solvent used for the test article was sterile distilled water. The highest concentration of the test article assayed in the cytotoxicity test was 5000 μg base/ml. The dosing solutions were adjusted to compensate for the mole fraction (0.825 free base) of the test article. The absence of second division metaphase cells in the solvent control cultures necessitated a repeat of the S-9 activated portion of the preliminary toxicity test. Only data from the repeated assay is contained in this report. The test article was soluble in solvent at a stock concentration of 500 mg base/ml, and soluble upon addition to treatment medium at all concentrations tested. However, after the 4 hour incubation at 37°C$\pm$1°C the test article, in the S-9 activated culture flasks only, was visible on the cell monolayer at the 1500 and 5000 μg base/ml concentrations. The osmolality of the highest concentration tested, 5000 μg base/ml, was 334 and 331 mOsm/kg in the nonactivated and S-9 activated test systems, respectively. The osmolality of the solvent (sterile distilled water) was 302 and 292 mOsm/kg in the

nonactivated and S-9 activated test systems, respectively. The pH of the test article was adjusted to approximately 7 in treatment medium using 1 N NaOH.

In the preliminary toxicity test complete mitotic inhibition was observed at dose levels of 150 μg base/ml and above in the nonactivated test system, and at dose levels of 50 μg base/ml and above in the S-9 activated test system (Tables 1 and 2). To ensure at least a 50% reduction in mitotic activity, 120 μg base/ml and 6 μg base/ml were selected as the highest dose levels for testing in the nonactivated and S-9 activated portions of the chromosome aberration assay, respectively. The chromosome aberration assay was conducted at dose levels of 8, 15, 30, 60, 90, and 120 μg base/ml in the absence of metabolic activation; and at 0.4, 0.8, 1.5, 3, and 6 μg base/ml in the presence of an Aroclor-induced S-9 activation system. Due to excessive toxicity observed in the nonactivated test system, this portion of the chromosome aberration assay was repeated at the following doses: 2, 4, 8, 15, 30, and 60 μg base/ml. Only data from the repeat nonactivated chromosome aberration assay are contained in this report. Extensive cell cycle delay was observed in the toxicity studies. Therefore, for the chromosome aberration assay, metaphase cells were collected at 24 hours after initiation of treatment in order to assure microscopic evaluation of first-division metaphase cells.

The activity of WR6026 Dihydrochloride in the induction of chromosome aberrations in CHO cells when treated in the absence of an exogenous source of metabolic activation is presented by treatment flask in Table 3 and summarized by group in Table 5. The test article was soluble in solvent at a stock concentration of 6 mg base/ml and soluble in treatment medium at all dose levels tested. Toxicity (mitotic inhibition) was approximately 97% at 30 μg base/ml, the highest test concentration evaluated for structural chromosome aberrations. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control ($p > 0.05$, Fisher's exact test). The percentage of aberrant cells in the MMC group was 51% ($p \leq 0.01$, Fisher's exact test).

The activity of WR6026 Dihydrochloride in the induction of chromosome aberrations in CHO cells when treated in the presence of an S-9 reaction mixture is presented by treatment flask in Table 4 and summarized by group in Table 5. The test article was soluble in solvent at a stock concentration of 0.6 mg base/ml and in treatment medium at all dose levels tested. Toxicity (mitotic inhibition) was 89% at 6 μg base/ml, the highest test concentration evaluated for structural chromosome aberrations. The percentage of cells with structural aberrations at the 3 μg base/ml dose level was statistically increased above that of the solvent control ($p \leq 0.05$, Fisher's exact test). However, the Cochran-Armitage test was negative for a dose trend and no statistically significant increase in the percentage of cells with structural aberrations was observed at this dose level relative to the untreated control ($p > 0.05$, Fisher's exact test). In addition, the percentage of aberrant cells seen at this dose level was within the range of the historical solvent control. Therefore, this observation was not considered to be biologically significant. The percentage of aberrant cells in the CP group was 37% ($p \leq 0.01$, Fisher's exact test).

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, WR6026 Dihydrochloride was concluded to be negative in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

TABLE 1
PRELIMINARY TOXICITY TEST USING WR6026 Dihydrochloride IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[4] (AGT)
Water	7.2		0	100	0	12.0
WR6026 Dihydrochloride						
0.5 µg base/ml	6.8	-6	1	99	0	12.1
1.5 µg base/ml	7.8	8	1	99	0	12.1
5 µg base/ml	8.4	17	1	99	0	12.1
15 µg base/ml	8.6	19	4	96	0	12.2
50 µg base/ml	5.8	-19	73	27	0	18.9
150 µg base/ml	0.0	-100	0	0	0	--
500 µg base/ml	0.0	-100	0	0	0	--
1500 µg base/ml	0.0	-100	0	0	0	--
5000 µg base/ml	0.0	-100	0	0	0	--

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.
[4]Average Generation Time:

$$\frac{\text{24 hours of BrdU exposure}}{[(1 \times \text{frequency M1 cells}) + (2 \times \text{frequency M2 cells}) + (3 \times \text{frequency M3 cells})]}$$

TABLE 2
PRELIMINARY TOXICITY TEST USING WR6026 Dihydrochloride IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[4] (AGT)
Water	5.8		7	93	0	12.4
WR6026 Dihydrochloride						
0.5 µg base/ml	9.6	66	20	80	0	13.3
1.5 µg base/ml	4.6	-21	93	7	0	22.4
5 µg base/ml	2.0	-66	98	2	0	23.5[5]
15 µg base/ml	0.0	-100	100	0	0	24.0[6]
50 µg base/ml	0.0	-100	0	0	0	--
150 µg base/ml	0.0	-100	0	0	0	--
500 µg base/ml	0.0	-100	0	0	0	--
1500 µg base/ml	0.0	-100	0	0	0	--
5000 µg base/ml	0.0	-100	0	0	0	--

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.
[4]Average Generation Time:

$$\frac{24 \text{ hours of BrdU exposure}}{[(1 \times \text{frequency M1 cells}) + (2 \times \text{frequency M2 cells}) + (3 \times \text{frequency M3 cells})]}$$

[5]AGT values were generated from a total of 64 metaphase cells.
[6]AGT values were generated from a total of 3 metaphase cells.

TABLE 3
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR6026 Dihydrochloride IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
					24 Hour Harvest							
Untreated cells	A	5.0	50	4	0	1	0	0	0	1	0	0.040
	B	5.4	50	2	0	0	0	0	1	0	0	0.020
Water	A	6.4	50	6	1	1	0	1	0	1	0	0.060
	B	6.6	50	4	0	2	0	0	0	0	0	0.040
WR6026 Dihydrochloride												
4 µg base/ml	A	6.0	50	0	0	0	0	0	0	0	0	0.000
	B	6.4	50	2	2	1	0	0	0	0	0	0.020
8 µg base/ml	A	3.8	50	0	0	0	0	0	0	0	0	0.000
	B	3.2	50	2	0	0	0	1	0	0	0	0.020
15 µg base/ml	A	1.6	50	4	0	1	0	0	1	0	0	0.040
	B	1.4	50	2	0	0	0	1	0	0	0	0.020
30 µg base/ml	A	0.2	27	7	0	0	0	2	0	0	0	0.074
	B	0.2	5	0	0	0	0	0	0	0	0	0.000
MMC, 0.08 µg/ml	A	3.6	50	50	0	13	7	10	1	0	0	0.620
	B	4.4	50	52	2	22	7	12	0	2	0	0.860

[1]CHO cells were treated for 24 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 4
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR6026 Dihydrochloride IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations						Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
					Chromatid-type[4]			Chromosome-type[5]				
					Gaps	Breaks	Exch	Breaks	Dic	Ring		
					24 Hour Harvest							
Untreated cells	A	6.2	50	2	0	0	0	1	0	0	0	0.020
	B	5.8	50	0	0	0	0	0	0	0	0	0.000
Water	A	7.4	50	0	0	0	0	0	0	0	0	0.000
	B	5.8	50	0	0	0	0	0	0	0	0	0.000
WR6026 Dihydrochloride												
0.8 µg base/ml	A	6.8	50	2	2	1	0	0	0	0	0	0.020
	B	5.2	50	2	1	1	0	0	0	0	0	0.020
1.5 µg base/ml	A	5.4	50	0	0	0	0	0	0	0	0	0.000
	B	4.2	50	2	1	1	0	0	0	0	0	0.020
3 µg base/ml	A	3.2	50	6	1	4	0	2	0	0	0	0.120
	B	2.6	50	4	1	1	0	1	0	0	0	0.040
6 µg base/ml	A	0.4	50	0	0	0	0	0	0	0	0	0.000
	B	1.0	50	0	0	0	0	0	0	0	0	0.000
CP, 10 µg/ml	A	2.4	50	30	6	8	14	1	0	0	0	0.460
	B	2.6	50	44	3	8	19	2	0	0	0	0.580

[1]CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 5
SUMMARY

Treatment	S-9 Activation	Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	24	5.2	100	0.030 ± 0.171	3
Water	-	24	6.5	100	0.050 ± 0.219	5
WR6026 Dihydrochloride						
4 µg base/ml	-	24	6.2	100	0.010 ± 0.100	1
8 µg base/ml	-	24	3.5	100	0.010 ± 0.100	1
15 µg base/ml	-	24	1.5	100	0.030 ± 0.171	3
30 µg base/ml	-	24	0.2	32	0.063 ± 0.246	6
MMC, 0.08 µg/ml	-	24	4.0	100	0.740 ± 0.906	51**
Untreated	+	24	6.0	100	0.010 ± 0.100	1
Water	+	24	6.6	100	0.000 ± 0.000	0
WR6026 Dihydrochloride						
0.8 µg base/ml	+	24	6.0	100	0.020 ± 0.141	2
1.5 µg base/ml	+	24	4.8	100	0.010 ± 0.100	1
3 µg base/ml	+	24	2.9	100	0.080 ± 0.394	5*[3]
6 µg base/ml	+	24	0.7	100	0.000 ± 0.000	0
CP, 10 µg/ml	+	24	2.5	100	0.520 ± 0.870	37**

[1]Severely damaged cells were counted as 10 aberrations.
[2]*, p≤0.05; **, p≤0.01; Fisher's exact test.
[3]Not statistically significant when compared to the untreated control.

REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol 4. Plenum Press, New York.

Perry, P., and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

APPENDIX I

Study Protocol

PROTOCOL AMENDMENT I

SPONSOR:	**Toxicology Research Laboratory**
TEST ARTICLE I.D.:	**WR6026 Dihydrochloride**
MA STUDY NO:	**G94BT87.330 (Protocol No.: SPGT330)**
SPONSOR PROJECT NO.:	**UIC-14**
PROTOCOL TITLE:	**Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

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1. **LOCATION:** Page 1, § 2.4;

   AMENDMENT: The Sponsor Project Number will be amended to UIC-14.

   REASON FOR THE AMENDMENT: Sponsor request.

APPROVALS:

Patrick T. Cimino — STUDY DIRECTOR — 1-3-95 — DATE

SPONSOR REPRESENTATIVE — 1/11/95 — DATE

MICROBIOLOGICAL ASSOCIATES, INC.

PROTOCOL AMENDMENT II

SPONSOR:	**Toxicology Research Laboratory**
TEST ARTICLE I.D.:	**WR6026 Dihydrochloride**
MA STUDY NO:	**G94BT87.330 (Protocol No.: SPGT330)**
SPONSOR PROJECT NO.:	**UIC-14**
PROTOCOL TITLE:	**Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

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1. **LOCATION:** Page 2 of 9, § 7.0; Experimental Design and Methodology

   AMENDMENT: Amend the fourth sentence to read "In order to ensure evaluation of first-division metaphase cells, the cell collection time may be delayed up to 24 hours in the event of cell cycle delay associated with the test article."

   REASON FOR THE AMENDMENT: Cell cycle kinetics data generated in the preliminary toxicity study indicates that the optimum cell collection time is 24 hours after initiation of treatment. This time point will allow analysis of first-division post-treatment metaphase cells.

2. **LOCATION:** Page 5 of 9, § 7.8; Treatment of Target Cells

   AMENDMENT: Amend the second sentence of the second paragraph to read "Treatment will be extended up to 24 hours in those dose levels with considerable cell cycle delay."

   REASON FOR THE AMENDMENT: To make consistent with changes in section 7.0.

3. **LOCATION:** Page 5 of 9, § 7.9; Collection of Metaphase Cells

   AMENDMENT: Amend the second sentence of the first paragraph to read "Cell fixation will not extend beyond 24 hours after initiation of treatment."

   REASON FOR THE AMENDMENT: To make consistent with changes in section 7.0.

APPROVALS:

Patrick T. [illegible] — STUDY DIRECTOR — 2-21-95 — DATE

[illegible] — SPONSOR REPRESENTATIVE — 2/21/95 — DATE

MICROBIOLOGICAL ASSOCIATES, INC.

APPROVED
cc 12-14-94

MA Study Number: G94BT87.330

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1	Name:	Toxicology Research Laboratory
2.2	Address:	University of Illinois at Chicago Department of Pharmacology 1940 West Taylor Street Chicago, IL 60612-7353
2.3	Representative:	Barry S. Levine, D.Sc., D.A.B.T.
2.4	Sponsor Project #:	UIC-10. This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Article:		WR6026 Dihydrochloride (mole fraction = 0.825) Bottle No. BK01845
	Storage:		-15 to -20°C Tightly closed in a desiccator Protect from light
3.2	Controls:	Negative: Solvent: Positive:	Untreated Cells Test Article Solvent Mitomycin C (MMC) Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Division of Genetic and Cellular Toxicology
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Patrick T. Curry, Ph.D.

4.4 Associate Study Director: Donald L. Putman, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 12-19-94

5.2 Proposed Experimental Completion Date: 2-22-95

5.3 Proposed Report Date: 3-10-95

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing CHO cells to a minimum of four concentrations of the test article as well as negative and positive controls in duplicate cultures. In the nonactivated test system, treatment will be continuous up to the time of cell collection; in the S-9 activated test system, exposure will be for 4 hours. The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 10 hours after the initiation of treatment. In order to ensure evaluation of first-division metaphase cells, the cell collection time may be delayed up to 20 hours in the event of cell cycle delay associated with test article treatment. The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to determine the solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Vehicles compatible

with this test system, in order of preference, include but are not limited to deionized, distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The vehicle will be the solvent, selected in order of preference, that permits preparation of the highest stock concentration, up to 500 mg/ml.

7.2 Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon mitotic inhibition after treatment. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the limit of solubility but not to exceed 5 mg/ml. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 6-8 hours in the absence of S-9 and for 4 hours in the presence of S-9. Two hours after initiation of exposure, 5-bromo-2'-deoxyuridine (BrdU), will be added to the culture medium at a final concentration of 0.01 mM. Following a 24 hour growth period in medium containing BrdU, with Colcemid® (0.1 μg/ml) present for the last 2 hours, the cells will be harvested by trypsinization. Metaphase preparations will be made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides will be evaluated for the percentage of first-, second- and third-plus-subsequent-division metaphase cells per 100 cells scored for determination of the test article effect on cell cycle kinetics. The average generation time (AGT) of each treatment condition will be calculated. The percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group.

Whenever possible, the high dose will be selected to give at least 50% toxicity (mitotic inhibition). At least three additional dose levels will be included in the assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, if excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if osmolality of the treatment medium is excessive, the Sponsor will be consulted prior to dose selection. The AGT will be used to determine the optimum harvest time for the cytogenetic study.

7.3 Frequency and Route of Administration

Target cells will be treated for 10 hours in the absence of S-9 (or up to 20 hours depending upon cell cycle delay) and for 4 hours in the presence of S-9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system.

7.4 Activation System

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The S-9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$) 6 mM potassium chloride (KCl), 1mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μl S-9 per ml serum free medium.

7.5 Controls

7.5.1 Untreated Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. For solvents other than water or medium, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 μg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 μg/ml as the positive control in the S-9 activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the nonactivated exposure or 5 ml S-9 reaction mixture for the S-9 activated exposure, to which will be added 50 μl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water or medium is used as the solvent. An untreated control consisting of cells in complete medium or S-9 reaction mixture will also be included.

In the nonactivated study, the cells will be treated for 10 hours at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. Treatment will be extended to up to 20 hours in those dose levels with considerable cell cycle delay. Treatment will continue until collection of metaphase cells.

In the S-9 activated study, the cells will be treated for 4 hours at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. After the treatment period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 10 hours after initiation of treatment or at a later time selected to represent the first division metaphase after initiation of treatment if the test article causes considerable cell cycle delay. Cell fixation will not extend beyond 20 hours after initiation of treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 μg/ml.

Cells will be harvested by trypsinization, collected by centrifugation, swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. One to two slides will be prepared from each treatment flask. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 $\pm$ 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads from each dose level (50 per duplicate flask) will be examined and scored for chromatid-type

and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ($\geq$ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented for the toxicity and aberration study. The AGT will be calculated for each treatment condition in the toxicity study as: AGT = (24 hours x 100)/[(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The number and types of aberrations found, the percentage of structurally damaged cells in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive

response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data.

Results presented will include , but not be limited to:

- cells used
- test conditions: composition of medium, CO_2 concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive and negative controls
- number of cell cultures
- number of metaphases analyzed (method for determination; data given separately for each culture)
- mitotic index
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, MD, 20850, in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill EPA and OECD test guidelines.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Yes .

If so, to which agency or agencies? FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes . If so, indicate which doses and volume will be required.

_____high only;

_____high and low only;

X all;

volume required 10 ml (if possible) .

The dosing solutions will be frozen and sent to UIC for analysis.

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

14.0 APPROVAL

Barry S. Levine 11/1/94

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) Date

G Schieferstein 11/10/94

Dr. George Schieferstein (US Army Contracting Officer's Representative) Date

Patrick T. Curry 12-13-94

MA Study Director Date

APPENDIX II

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS

STRUCTURAL ABERRATIONS
HISTORICAL CONTROL VALUES
1992-1994

NONACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.2%	1.4%	36.7%
Standard Deviation	1.1%	1.2%	22.8%
Range	0.0% to 5.5%	0.0% to 6.0%	8% to 100.0%

S-9 ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.7%	1.7%	42.7%
Standard Deviation	1.3%	1.2%	24.0%
Range	0.0% to 5.0%	0.0% to 5.0%	7.5% to 100.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, toluene, CMC, PBS, 2% DMSO in saline, CSEP Buffer, Citrate Buffer, culture medium, 1% Human Albumin, Extraction Blanks
[2]Positive control for nonactivated studies, triethylenemelamine, (TEM), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and Mitomycin C (MMC).
[3]Positive control for S-9 activated studies, cyclophosphamide, (CP), and benzo(α)pyrene, (B[α]P).